BIOSYNTHESIS OF POLY(3-HYDROXYBUTYRATE) (PHB) BY *Cupriavidus necator* H16 FROM JATROPHA OIL AS CARBON SOURCE

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ABSTRACT

The increasing non-biodegradable plastic waste materials have created critical need in finding a better replacement to the currently available conventional plastic. Researches have shown great interest in the production of polyhydroxyalkanoate (PHA) biopolymer from bacterial fermentation. Although successful attempts have been made in producing these short to medium-chain length biopolymers, there are still problems in terms of yield and cost effectiveness that needs to be resolved. Poly(3-hydroxybutyrate) (PHB), a homopolymer of PHA is one particular example of bioplastic that are naturally produced by bacteria like *Cupriavidus necator* sp. By using plant oils such as jatropha oil as an alternative, a higher yield of PHB can be obtained and thus reducing the overall production cost of the biopolymers. In this study, *Cupriavidus necator* H16 was used to synthesize PHB by using jatropha oil as its sole carbon source. Different variables mainly jatropha oil and urea concentrations, and agitation speed were investigated to determine the optimum condition for microbial fermentation in batch culture. Based on the results, the highest cell dry weight and PHB concentration of 20.1 g/L and 15.5 g/L respectively was obtained when 20 g/L of jatropha oil was used along with 1 g/L of urea at 200 rpm of agitation speed. Ethanol was used as external stress factor and the addition of 1.5% (v/v) ethanol at 38 h had a positive effect with a high PHB yield of 0.987 g PHB/g jatropha oil. The kinetic studies for cell growth rate and PHB production were conducted and the data were fitted with Logistic and Leudeking-Piret models. The rate constants were evaluated and the theoretical values were in accordance with the experimental data obtained. Optimization through Response Surface Methodology (RSM) at the condition of 0.9 g/L urea, 23.6 g/L jatropha oil and 251 rpm agitation speed resulted in 5% increase in PHB concentration to 17.92 g/L compared to the previously obtained PHB concentration of 17.05 g/L. The present work has succeeded in obtaining a high yield of PHB from an inexpensive raw material.

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LIST OF SYMBOLS

M_N	Average molecular weight
M_w	Molecular weight
P	Product concentration (g/L)
P_0	Initial product concentration (g/L)
T_d	Thermodegradation temperature
T_g	Glass transition temperature
T_m	Melting temperature
t	Time (h)
t_m	Time when maximum cell concentration is reached (h)
x	Cell concentration (g/L)
x_m	Maximum cell concentration (g/L)
x_0	Initial cell concentration (g/L)
α	Growth associated constant (g/g)
β	Non-growth associated constant (g g^{-1} h^{-1})
μ_m	Maximum specific cell growth rate (h^{-1})

LIST OF ABBREVIATIONS

BOD	Biological oxygen demand
CDW	Cell dry weight
COD	Chemical oxygen demand
CPKO	Crude palm kernel oil
CPO	Crude palm oil
FDA	Food and Drug Administration
GC	Gas chromatography
HBME	Hydroxybutyric methyl esters
HHx	Hydroxyhexanoate
HPLC	High Performance Liquid Chromatography
HV	Hydroxyvalearate
mcl-PHA	Medium chain length PHA
MMC	Mixed microbial concortia
NADPH	Nicotinamide adenine dinucleotide phosphate
PHA	Polyhydroxyalkanoate
PHB, P(3HB)	Poly-3-hydroxybutyrate
PLA	Polylactic acid
phaA	β-ketothiolase
phaB	Acetoacetyl–CoA reductase
phaC	PHA synthase
PO	Palm oil
POME	Palm oil mill effluent
P&G	Procter & Gamble
PTFE	Polytetrafluoroethylene

RSM	Response Surface Methodology
scl-PHA	Short chain length PHA
SPKO	Saponified palm kernel oil
TCA	Tri-carboxylic acid cycle
SBR	Sequencing batch reactors
USD	US Dollars
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Undeniably, petroleum-based synthetic plastics offer a wide range of industrial and domestic applications due to their convenience and durability. The short-term convenience of using and throwing these conventional plastics have created major problem since they cannot be degraded naturally in the environment. These plastic wastes pile up in landfills and take hundreds of years to degrade (Wurpel et al., 2011). The situation is made worse when these plastic wastes are thrown carelessly into the ocean, endangering marine life. Apart from that, the diminishing worldwide petroleum resources compels for a better alternative for petroleum-based plastics. Based on the report by US Energy Information Administration, about 191 million barrels of liquid petroleum gases and natural gas liquids were used in the United States to make plastic products in the plastic materials and resins industry which is equivalent to about 2.7% of total U.S. petroleum consumption (US Enery Information Administration, 2010).

The current concerns over the increasing usage of non-biodegradable plastics and its impact to the nature have pushed researchers to develop bioplastics that are biodegradable and environmental-friendly. Biodegradable plastics have the potential to replace conventional plastics as they are environmentally-friendly. These biopolymers can be synthesized from renewable raw materials and thus reducing the greenhouse gas effect. Polyhydroxyalkanoates (PHA) and polylactic acid (PLA) are an example of biodegradable plastic that are produced by fermentation using agricultural products and microorganisms (Tokiwa et al., 2009).

This research emphasis is on PHA biopolymers which are produced by various microbes under nutrient-limiting conditions (e.g.: limitation of sodium and phosphorus) but with an excess of carbon source (Luengo et al., 2003). The bacterial strains used for PHA biosynthesis are categorized based on the culture medium used during fermentation. The first group, consisting of microbes such as *Cupriavidus necator*, *Protomonas extorquens* and *Protomonas oleovorans*, requires the presence of excess carbon source and limitation of nutrients like nitrogen or phosphorus. Meanwhile, the second group can accumulate PHA during growth phase itself at a nutrient-sufficient condition. Bacterial strain in this group include *Alcaligenes latus*, a mutant strain of *Azotobacter vinelandii* and recombinant *E. coli* harboring the PHA biosynthetic operon of *C. necator* (Khanna and Srivastava, 2005; Lee, 1996).

The specific biopolymer synthesized in this research was poly-3-hydroxybutyrate (PHB). PHB, the most common type of PHA, can be accumulated by various microorganisms. These short-chain length biopolymers have been studied and characterized comprehensively (Madison and Huisman, 1999). This research focusses on elucidating the influence of various process parameters to determine the optimum condition for the bacterial fermentation of *Cupriavidus necator* H16 to produce PHB by using jatropha oil as its main carbon source.

1.2 PROBLEM STATEMENT

Although researches on PHB synthesis from microorganisms are abundant, the large-scale manufacture of PHB-based bioplastics is still limited due to its high production cost. Since the raw material cost is one of the major elements in the production of PHB, a good choice of the feed substrate may reduce significantly the overall PHB production cost. At present, large-scale production of PHB uses sugars like fructose and glucose as their carbon source. These sugars at USD 0.50/kg are expensive (Choi and Lee, 1997) and on top of that, they also give a low yield of PHB (Kahar et al, 2004). Thus, in order to make the biopolymer production a more practical approach, an alternate raw material that is cost-effective and at the same time does not affect the yield of PHB should be used.

One better way of achieving this goal is to substitute the carbon source into a more cost efficient ones that are derived from plant oils. Plant oils such as jatropha oil are known to give a theoretical yield of PHA of over 1.0 g-PHA per g-plant oils used compared to glucose which only gives a yield of 0.32–0.48 g-PHA/g-glucose (Kahar et al, 2004). Thus, with the usage of jatropha oil as its carbon source, the production cost of PHB-based biopolymers can be radically reduced without compromising the PHB yield. Ng et al. (2010) have reported convincing results of 13.1 g/L cell dry weight and 11.4 g/L of PHB from the bacterial fermentation of *Cupriavidus necator* H16 using 12.5 g/L jatropha oil as their sole carbon source. Various nitrogen sources were studied by Ng et al. (2010) and urea at 0.54 g/L was concluded as the most suitable nitrogen source that gives high PHB accumulation.

The outstanding result had encouraged us to explore the possibility of further enhancing the PHB yield by studying the influence of various process parameters on PHB accumulation. Mainly, we examined the effect of external stress factor on the bacterial growth and PHB accumulation. Research on the optimization and kinetic studies on production of PHB from *Cupriavidus necator* sp. by using jatropha oil were also limited. Therefore, additional research concerning these aspects was done to improve the overall understanding on PHB production from jatropha oil as carbon source.

1.3 RESEARCH OBJECTIVES

The main objective of this research is to study the reaction kinetics of the production of PHB from *Cupriavidus necator* sp. by using jatropha oil as its sole carbon source. Different aspects such as the agitation speed, oil and urea concentration and stress factor effect were analyzed to determine the best condition for PHB production. These conditions were optimized further to increase the yield of PHB and mathematical models were be developed for cell growth and PHB accumulation. The specific objectives of this study include:-

1. To study the effect of various process parameters (agitation speed, oil and urea concentration etc.) in the production of PHB from *Cupriavidus necator* sp. by using jatropha oil as its main carbon source.

2. To study the kinetics and develop the corresponding mathematical model of PHB production and conduct optimization of PHB by using Response Surface Methodology (RSM).

1.4 RESEARCH SCOPE

The study plan focuses on the production of PHB by using jatropha oil as carbon source. In order to achieve the above stated objectives, the following scope of research has been identified:

1. The bacteria *Cupriavidus necator* sp. were fermented in shake flask and the study of different variables (agitation speed, oil and urea concentration, stress factor effect) were conducted.

2. Quantitative analysis were done on the biopolymer produced by using Gas-Chromatography (GC) analysis to determine the PHB concentration in cells.

3. Subsequently, the research were expanded to study the optimization of the biopolymer synthesis using Response Surface Methodology (RSM) to gain the optimum conditions for the highest PHB concentration in cells.

4. The kinetic studies for cell growth rate and PHB production were conducted and the data were fitted with Logistic and Leudeking-Piret models. The rate constants were evaluated and the data obtained were compared with the calculated theoretical values.

1.5 SIGNIFICANCE OF RESEARCH

Through this research, it is believed that a higher yield of PHB can be obtained by optimizing the variable conditions and by doing kinetic studies on the biopolymer synthesis. Moreover, using jatropha oil as the carbon source for PHA production may lessen the overall production cost considerably and thus making it a more feasible approach for large-scale production. In addition, jatropha oil has an added advantage of being non-edible oil. Therefore, utilizing it for bioplastic production would not interfere with the existing global food shortage issue.

1.6 THESIS OVERVIEW

This thesis comprises of five main chapters. Chapter 1 discloses the introduction and Chapter 2 has a detailed review on literatures related to polyhydroxyalkanoate (PHA). Meanwhile Chapter 3 discusses the methodology, apparatus and experimental equipment used throughout this research. Chapter 4 holds comprehensive discussions on the experimental results obtained and Chapter 5 discusses the overall summary and recommendations for future work. References and appendices are also included for better understanding of the research.

CHAPTER 2

LITERATURE REVIEW

This chapter mainly consists of findings from previous researches with regards to production of PHB. Detailed discussions on the properties, synthesis mechanism, and the comparison of various bacterial strains and carbon sources used in the production of PHB and its homopolymers were presented in the following sections.

2.1 INTRODUCTION

Presently, the diminishing global petroleum resources have created urgent need towards finding sustainable alternate sources for value-added chemicals. In addition, petroleum-based plastics are known to be hazardous given that they cannot be degraded naturally in the environment. Thus, a more enhanced approach would be to implement the usage of biodegradable plastics that are cheap and have similar properties to the commercial plastics.

In general, bioplastics are defined as a kind of biomaterial that are cultivated under specific nutrient and environmental conditions by using a variety of microorganism and carbon substrate as their raw material. These polymers are used as storage materials by microbes to survive under nutrient-deficient condition (Madison and Huissman, 1999). Although numerous researches have been done with regards of bioplastics, the large-scale production is still limited due to the low productivity and also high manufacturing cost. As for the past few years, there are a few types of bioplastics available such as starch and cellulose based plastics, polylactic acid (PLA) plastics and also polyhydroxyalkanoate (PHA) based polyester plastics.

PHAs are completely biodegradable and biocompatible polymers with properties such as thermoplastic, elastomer, insoluble in water and also non-toxic in nature (Ng et. al, 2010). These polyesters have characteristics similar to those of polyethylene and polypropylene, and can therefore be used as a substitute to conventional plastics. Apart from that, they are also degraded completely under aerobic and anaerobic conditions by microorganisms (Luengo et al., 2003), and thus putting an end to the increasing non-biodegradable municipal solid waste problems. Figure 2.1 shows the general structure of PHAs.

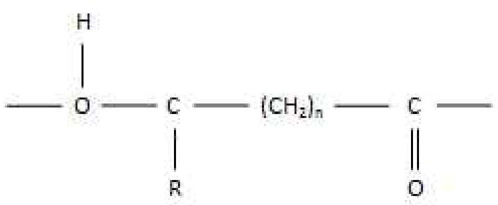

Figure 2.1: General structure of polyhydroxyalkanoates

Source: Volova (2004)

PHAs have a variety of usage in the industries due to the similarities of physical and thermal properties between commercial plastics and bioplastics produced from PHA polymers. PHA bioplastics have great potential to be used as packaging films in bags, containers and paper coatings. These polymers can also be used as a replacement of the regular commercial plastic to manufacture disposable items such as razors, utensils, cosmetic containers and so on. The comparison between physical and mechanical properties of PHA and polypropylene, a common synthetic plastic, is shown in Table 2.1.

Table 2.1: Physical and Mechanical Properties of PHA

Properties	PHA	Polypropylene
Molecular Weight, M_w x 10^4	10 – 1000	-
Melting Temperature, T_m (^{0}C)	60 – 177	176
Glass Transition Temperature, T_g (^{0}C)	-50 – 4	-10
Thermodegradation Temperature, $T_{d(5\%)}$ (^{0}C)	227 – 256	-
Young's Modulus (GPa)	0.7 – 3.5	1.7
Elongation at Break (%)	2 – 1000	400
Tensile Strength (MPa)	17 – 104	34.5

Source: Chen (2009) and Ojumu et al. (2004)

Generally, PHAs can be divided into two main groups according to the number of carbon atoms in the monomeric units. These include short chain length PHAs (scl-PHA) which consist of 3-5 carbon atoms in the constituting monomeric unit of the polymer and also medium chain length PHAs (mcl-PHA) consisting of 6-14 carbon monomers (Ojumu et al., 2004). One particular example of PHA is poly(3-hydroxybutyrate) (PHB) which is a homopolymer that contains monomers of 3-hydroxybutyrate. The molecular structure of PHB is displayed in Figure 2.2. PHB has crystalline properties with a melting point of around 170 ^{0}C (Kulkarni et al., 2010). With degradation temperature (185^{0}C) recorded just slightly above its melting temperature, PHB has an unstable nature during its melting stage (Ojumu et al., 2004). Furthermore, its crystallinity, hardness and brittleness forces it to be used only as specialty plastics for certain types of industries. Thus, to overcome these problems, several attempts have been made by incorporating comonomers such as 3-hydroxyvalearate (HV) and 4-hydroxybutyrate (HB) into PHB to reduce its brittleness.

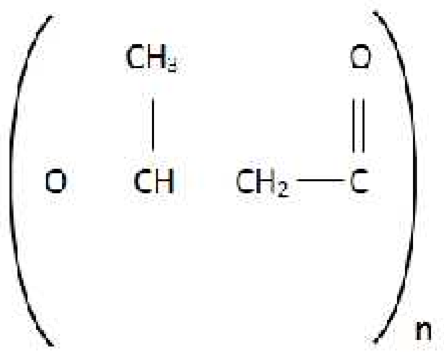

Figure 2.2: Molecular structure of PHB, n can range from 100 to several thousands

Source: Salakkam (2012)

2.2 PHA APPLICATION

PHA based biopolymers have garnered immense interest due to their biodegradability and biocompatibility. The following subtopics discuss the current and future applications of PHA.

2.2.1 As Packaging Material and Disposable Items

The similarity of PHA and other synthetic plastics renders it beneficial to be used as packaging films mainly in bags, containers and paper coatings. Likewise, its biodegradability makes it a suitable choice as a substitute for regular plastic disposable items such as razors, utensils, diapers, feminine hygiene products and cosmetic containers like shampoo bottles and cups (Reddy et al., 2003). Procter & Gamble (P&G, USA) had developed, Nodax ™, a bioplastic based on polyhydroxybutyrate-hexanoate (PHB-HHX). Nodax ™ can be used to manufacture a variety of plastic materials including packaging, laminates and coatings, and nonwoven fibers (Noda et al., 2010). Figure 2.3 portrays the various products based on Nodax ™ bioplastic.

Figure 2.3: Various products from Nodax ™ bioplastic

Source: Paliakoff and Noda (2004)

2.2.2 In Medical and Pharmaceutical Industries

The non-toxicity and biocompatibility of PHB based biopolymers offers great potential to be used in medical and pharmaceutical industries. Upon degradation, these bioplastics will be degraded into D-3-hydroxybutyrate is a common intermediate metabolic compound in all higher organisms (Lee, 1996). PHA biopolymers can be used as surgical pins, sutures, and swabs, wound dressing, bone replacements and plates, blood vessel replacements in healthcare industries (Reddy et al., 2003). The main advantage of using PHA bioplastic in surgical implantation is its biodegradability which enables it to degrade naturally without the need for surgical removal of the implant. In the pharmaceutical industry, PHB is used in drug delivery system as a matrix material for slow release drugs and in vitro cell cultures (Suriyamongkol et al., 2007). Nevertheless, their applications in the pharmaceutical and medical fields are still restricted due to their slow biodegradation and high hydraulic stability in sterile tissues (Wang and Bakken, 1998).

2.2.3 In Agricultural Industries

The agricultural industry may offer a vast array of application which includes seed encapsulation, encapsulation of fertilizers and protective material for crops in the form of biodegradable plastic films. The biopolymer P(3HB-3HV) could be used in the controlled release of insecticides for crops. The commercially available Nodax ™

bioplastic could also be used as coating for urea fertilizers in rice fields. The biopolymer, which can be degraded anaerobically, can also be used as herbicides and insecticides (Yogesh et al., 2012).

2.3 COMMERCIALISATION OF PHA

Although PHA has great potential to be used as a substitute for conventional plastics, its large scale production is still restricted due to its cost effectiveness. As mentioned earlier, the cost of raw material and also the recovery process play a major role in the overall production cost of PHA biopolymer. Numerous researches were conducted to address this crucial problem so that PHA based biopolymer can be made commercially viable. Through constant research, the price for Biopol™ plastics was reduced from 16 USD/kg to 4 USD/kg. Nevertheless, the price is still expensive when compared to plastics made from polypropylene and polyethylene (0.25 – 0.5 USD/kg) (Chandrasekharaiah, 2005). Biopol is produced industrially by bacterial fermentation of *Cupriavidus necator* with glucose as its carbon source. The annual production of Biopol was about 10,000 tonnes (Lee, 1996). In 1990, the product was successfully used for the marketing of German's hair care company, Wella's Sanara shampoo bottle (Chen, 2010). Table 2.2 presents a list of PHA producing companies around the world.

Table 2.2: Worldwide PHA producers

Type of PHAs	Company and origin	Trade name	Price (USD/kg)	Microorganism
PHB	Biocycles, Brazil	Biocycle	3.12-3.75 (2010)	*Alcaligenes* sp.
PHB	Biomer, Germany	Biomer™	3.75-6.25 (2010)	*A. latus*
PHB	Chemie Linz, Austria	-	-	-
PHB	Jiangsu Nan Tian, China	-	-	-
PHB	Mitsubishi Gas Chemical, Japan	Biogreen™	2.75 (2010)	Methanol utilising bacteria
PHB/PHV	Metabolix, USA	Biopol™	4 (2005)	Glucose utilizing mutant of *C. necator*
PHA/PHB/ PHO*	Metabolix, USA	Metabolix PHA	-	Recombinant *E. coli* K12
PHA copolymer	Meredian, USA	Nodax™	-	*Aeromonas caviae* and *C. necator*

*PHO – polyhydroxyoxanoate

Source: Salakkam (2012)

Currently, there are several brands of PHA that are available in the market. These PHAs are produced at a large scale by using sugar as their carbon source. Some examples of commercially produced PHAs include Biopol™ (copolymer of hydroxybutyrate (HB) and hydroxyvalerate (HV)), Biomer™ (homopolymer of HB), Nodax™ (copolymer of HB and hydroxyhexanoate (HHx)) and Biocycle™ (homopolymer of HB, copolymer of HB and HV) (Mumtaz et al., 2010). Tepha, a PHA bioplastic producing company, have succeeded in commercializing medical devices such as the Food and Drug Administration (FDA) approved PHA-based sutures. Nevertheless, usages of bioplastics from PHA are still limited mainly because their production cost are still very high when compared to petroleum-based polyesters. One of the major problems faced in reducing its production cost include selecting a relatively cheap but equally viable carbon source.